



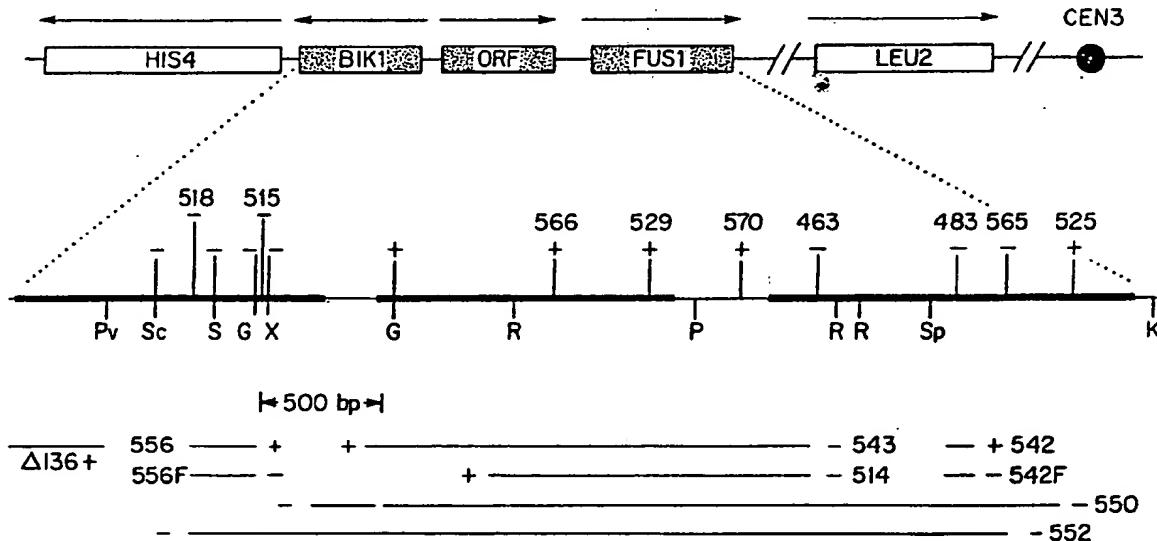
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(54) Title: PHEROMONE-INDUCIBLE YEAST PROMOTER



(57) Abstract

A yeast promoter inducible by the appropriate pheromone and a method of expressing a gene of interest in substantial quantities by placing it under the control of the inducible promoter. DNA encoding a protein of interest is fused or linked to a pheromone-inducible yeast promoter, such as the *FUS1* or the *FUS2* promoter, and the fusion is inserted onto a high copy vector; the resulting product is introduced into wild type yeast cells. Stimulation of these yeast cells by the appropriate pheromone results in induction of transcription of the yeast promoter and expression of the protein of interest in substantial quantities.

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PHEROMONE - INDUCIBLE YEAST PROMOTER

Description

Background

It is possible, using recombinant DNA technology, to
05 clone and express in bacteria and yeast a variety of
genes which are not present in such organisms as they
occur in nature. For example, procedures for cloning DNA
segments in E. coli, by inserting the DNA into a plasmid
or bacteriophage genome, are well established and
10 frequently used to isolate prokaryotic and eukaryotic
genes.

The yeast Saccharomyces cerevisiae (S. cerevisiae)
is also used as a host for heterologous gene expression
and protein secretion. Availability of techniques which
15 make it possible to introduce exogenous DNA into yeast
genomic DNA has made it possible to develop yeast strains
which produce and secrete foreign proteins such as alpha
interferon, epidermal growth factor, calf prochymosin and
beta-endorphin. Heterologous eukaryotic genes can be
20 expressed in S. cerevisiae if they are placed under the

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control of a yeast gene promoter region. Heterologous protein yields will be determined, at least in part, by the promoter chosen. As an example, the promoter from the yeast gene for phosphoglycerate kinase (PGK) has been 05 described as directing expression of heterologous genes with efficiency at least 500-fold greater than that evident when the TRP1 promoter is used. Mellor, J. et al., Gene, 33:215-226 (1985). Hitzeman and co-workers describe work to increase expression of the gene encoding 10 bovine growth hormone in yeast. The 5'-promoter region, translation signal and signal peptide sequences were replaced with yeast genomic DNA from similar regions. Hitzeman, R. A. et al., Nature, 295:717-722 (1981).

The type of vector chosen (e.g., a high-copy-number 15 2 micron plasmid-based shuttle vector, rather than an unstable high-copy-number ARS-based plasmid or a low-copy-number stable ARS/CEN plasmid) also affects yields of heterologous proteins.

The availability of yeast promoters which are 20 efficient in directing expression of a heterologous gene and whose activity can be regulated would be desirable.

Disclosure of the Invention

The present invention is based on the identification and isolation of two genes which are required for 25 efficient cell fusion during yeast conjugation. Transcription of these two genes has been shown to be greatly induced by the presence of the appropriate mating pheromone. Expression of a gene of interest placed under the control of the promoter of either of these two genes 30 has also been shown to be similarly induced by the presence of the appropriate mating pheromone. When the

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promoter-gene of interest combination is present on a high-copy vector in yeast cells stimulated by the appropriate mating pheromone, expression of the fusion product is greatly increased.

05 According to the present invention, DNA encoding a protein of interest is placed under the control of a yeast (e.g., S. cerevisiae) promoter whose transcription is induced by the appropriate mating pheromone. Induction of the yeast promoter occurs as a result of 10 stimulation by the appropriate mating pheromone.

According to the present invention, DNA encoding the protein of interest (DNA of interest) is fused or linked to the pheromone-inducible yeast promoter, using known techniques; the promoter-DNA of interest fusion is 15 inserted onto an appropriate vector; vectors containing the fusion are introduced into yeast cells (e.g., by transformation); and induction of transcription of the promoter is stimulated by the appropriate mating pheromone (i.e., alpha factor for a cells, a factor for 20 alpha cells). Induction of the yeast promoter in this manner also results in expression of the DNA of interest which is under its control, resulting in production of the protein encoded by the DNA of interest. If the promoter-DNA of interest fusion is inserted onto a high 25 copy vector (such as a high copy two micron vector), stimulation by the appropriate mating pheromone results in a significant increase in induction of the promoter-DNA of interest fusion and production of the encoded protein in substantial quantities. Thus, it is 30 possible to clone DNA (i.e., an entire gene or a gene segment) encoding a protein or polypeptide in yeast, with expression of the DNA being controlled by the inducible

promoter; the promoter's activity is, in turn, determined by the presence or absence of stimulation by the appropriate mating pheromone.

In one embodiment, a DNA fragment from either the 05 FUS1 gene or the FUS2 gene of S. cerevisiae, which includes the FUS1 promoter or the FUS2 promoter, respectively, is fused or linked, using techniques known to those skilled in the art, to DNA encoding a protein of interest. The DNA fragment of the FUS1 or FUS2 gene is a 10 5' segment or N terminal moiety. The yeast DNA fragment - DNA of interest fusion is inserted into a vector and the vector introduced into wild-type yeast cells. Upon stimulation of the yeast cells by the appropriate pheromone, induction of transcription of the yeast gene 15 fragment (5' segment) occurs, the DNA of interest is expressed and a fusion protein which includes the FUS1 (or FUS2) -encoded protein sequence and the protein or polypeptide encoded by the DNA of interest is produced.

For example, DNA from the FUS1 gene which includes 20 the FUS1 promoter and sequences encoding approximately the first 254 amino acids of FUS1 is fused to DNA encoding a protein or polypeptide of interest and the resulting combination inserted onto a high copy two micron vector (to produce a FUS1-DNA of interest 25 plasmid). The resulting plasmid is introduced into wild-type a cells, which are incubated with alpha factor. Alpha factor induces a-type cells to respond; induction of the FUS1 promoter leads to induction of transcription of the DNA of interest to which it is fused. The result 30 is expression of the FUS1-DNA of interest fusion protein (i.e., a fusion protein which is the FUS1-encoded

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protein and the protein encoded by the DNA of interest) at substantial levels.

05 DNA encompassing the promoter from either the FUS1 gene or the FUS2 gene can be fused to DNA of interest, incorporated into an appropriate vector and introduced into yeast cells, which are incubated with the appropriate mating pheromone. Incubation in this manner causes induction of transcription of the fusion which includes the FUS1 promoter or the FUS2 promoter and DNA 10 of interest and expression of the DNA, resulting in production of the encoded protein or polypeptide.

15 This approach can be used to produce proteins of interest in substantial amounts in yeast cells. The method of the present invention is particularly valuable because yeast cells containing the fusion can be grown up (cultured) passively and, after sufficient quantities of cells are produced, induced to express the fusion protein (containing the protein of interest) in substantial quantities. This is an important advantage because many 20 foreign proteins are toxic to yeast cells.

Brief Description of the Drawings

Figure 1 is a schematic representation of chromosome III of the yeast Saccharomyces cerevisiae, on which the BIK-FUS1 region adjacent to HIS4 is shown.

25 Figure 2 shows results of Northern hybridizations of total RNA isolated from yeast strains treated with alpha-factor or solvent minus alpha-factor.

30 Figure 3 are immunofluorescence micrographs of alpha-factor-induced spheroplasts fixed in formaldehyde and incubated with anti-beta-galactosidase antibody and FITC-conjugated anti-mouse Ig.

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Figure 4 presents the nucleotide sequence of the BIK1-FUS1 region. Position 1 corresponds to position -245 of the HIS4 gene.

05 Figure 5 presents the nucleotide sequence of the FUS2 gene and the deduced amino acid sequence. It includes approximately 500 nucleotides upstream and, thus, encompasses the hexameric repeats thought to be required for regulation.

Detailed Description of the Invention

10 The yeast S. cerevisiae can exist in a diploid or a haploid state; in the former, it has 17 pairs of chromosomes and, in the latter, a single set of chromosomes (one copy of each of the 17 chromosomes). Under favorable conditions, yeast cells exist in the 15 diploid state and multiply rapidly. Multiplication occurs through mitosis, the process by which a diploid cell duplicates its chromosome pairs and divides into a mother cell and a bud, each of which has two copies of every chromosome.

20 When conditions are unfavorable, yeast cells sporulate; cells stop dividing and undergo meiosis, with the result that they duplicate their chromosome pairs and divide into four cells, each having a set of chromosomes. These haploid cells are of either the alpha type or the a 25 type. They can reproduce by mitosis, but produce only haploids as a result. They can enter the diploid life cycle by conjugation, during which an alpha cell and an a cell fuse to form a diploid cell.

Conjugation in S. cerevisiae involves the fusion of 30 haploid cells of opposite mating type, followed by the fusion of nuclei to form a diploid. The zygote formed by

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this process buds off diploid cells capable of vegetative growth. Formation of the zygote requires the coordination of two processes--cell fusion and nuclear fusion. Both processes are initiated by mating 05 pheromones: a cells produce a-factor, to which alpha cells specifically respond, and alpha cells produce alpha-factor, to which a cells specifically respond. Duntze, W.D. et al., European Journal of Biochemistry, 35:357-365 (1973); Wilkinson, L.E. and J.R. Pringle, 10 Experimental Cell Research, 89:175-187 (1974).

Cells stimulated by the appropriate mating pheromone produce surface agglutinins (resulting in extensive clumping of conjugating cultures), arrest their cell-cycle at the G1 stage, and elongate to form a 15 discernible tip (a process dubbed "shmooing"). When the appropriate partners have achieved contact, presumably at the shmoo tip, the cells rapidly fuse. Cell fusion requires the degradation and/or reorganization of the cell wall and the fusion of the two plasma membranes.

20 The nuclei subsequently fuse within the dumbbell-shaped zygote formed and the resultant diploid nucleus begins a series of division cycles, each of which yields a new diploid nucleus that enters an emerging bud. Nuclear fusion is not a passive process; like cell fusion, it 25 requires potentiation by the mating factors. Curran, B.P.G. and B.L.A. Carter, Current Genetics, 10:943-945 (1986); Rose, M.D., et al., Molecular and Cellular Biology, 6:3490-3497 (1986).

Two genes involved in the initial zygote formation 30 (cell surface reorganization leading to cytoplasmic fusion) have been identified. They have been designated FUS1 and FUS2. Cloning of FUS1 and FUS2 revealed that

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they share some functional homology. That is, FUS1 on a high copy plasmid can partially suppress a FUS2 mutant; the opposite is also true. FUS1 is essentially unexpressed in vegetative cells, but is strongly induced 05 by incubation of haploid cells with the appropriate mating pheromone. When a cells are incubated with alpha factor, transcription of FUS1 and FUS2 is strongly induced. The same is true of FUS1 when alpha cells are induced by a factor. Mutations in these genes block the 10 conjugation process at a point following cell contact, preventing cytoplasmic fusion and, as a result, nuclear fusion. Genes containing these mutations have been designated fus, for cell fusion defective.

The expression of these FUS genes has been shown to 15 be activated by mating pheromones, an induction that leads to the appearance of the gene product at the tip of the shmoo. This localization suggests that the defect observed in matings between mutants is a direct result of the absence of the gene product, rather than a symptom of 20 some general metabolic defect. Investigation of the FUS1 gene has shown that it is not expressed in vegetative haploid a and alpha cells and diploid a/alpha cells and achieves high levels only in the presence of mating pheromones, suggesting that it functions exclusively 25 during the actual conjugation process.

Induction of the level of FUS1 mRNA was assessed to determine whether its expression is altered during conjugation. Assessment of FUS1 transcription is described in Example 1.

30 Similarly, when cells containing the FUS1 gene or a portion including the FUS1 promoter fused to DNA of interest are exposed to the appropriate mating pheromone

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(alpha factor), transcription of the FUS1 promoter is greatly induced, and leads to induction of transcription of the DNA which is under the control of the FUS1 promoter. Pheromone induction has been demonstrated by 05 expression of a fusion protein encoded by a FUS1-LACZ fusion which has two components: a FUS1 fragment including the FUS1 promoter region and sequences encoding approximately the first 254 amino acids of FUS1 and the gene encoding beta-galactosidase. Exposure of wild-type 10 a cells containing the fusion on a high-copy 2 micron vector resulted in at least 1000-fold induction of beta-galactosidase. When single-copy derivatives of this fusion were used, an equivalent induction ratio was observed. Pheromone induction has also been demonstrated 15 by expression of a fusion protein encoded by a FUS1-SUC2 fusion, which includes a FUS1 fragment and the gene encoding invertase which lacks the normal signal sequence for invertase.

As a result of the work described herein, two highly 20 inducible yeast promoters have been identified and can be used for achieving substantial levels of expression for a DNA sequence or gene of interest in yeast. The DNA sequence or gene of interest can encode a polypeptide or protein of interest (i.e., one whose expression is 25 desired). Proteins and polypeptides of interest include those not normally expressed in yeast cells (i.e., heterologous proteins), as well as proteins normally expressed in yeast cells but at lower levels than can be achieved through use of the method of the present 30 invention. As used herein, the term protein is meant to include proteins and polypeptides.

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Recombinant DNA techniques known to those in the art are used to obtain the necessary genetic material and to introduce it into yeast cells, which are subsequently cloned. Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

The DNA encoding the protein or polypeptide of interest can be isolated by obtaining mRNA of the desired gene or gene segment. For example, double-stranded DNA can be produced from the mRNA by conventional means. First, a complementary copy of the DNA is made from the mRNA, using conventional RNA technology; the RNA is subsequently removed by breaking the strands, using art-recognized methods. The cDNA is then made double-stranded; E. coli DNA polymerase I can be used for this step.

Synthetic linkers can be added to both ends of the double-stranded DNA (e.g., by using HindIII or EcoRI synthetic oligonucleotide linkers). cDNA having linkers at both ends is introduced into the unique site of a plasmid, which has been cut with the appropriate restriction enzyme. DNA having linkers at both ends can, alternatively, be introduced into a virus or cosmid. For example, pBR322, pM89 or lambdaGTWES can be used for this purpose.

Transformation of bacteria with the ligation products will result in transformation of only a fraction of the cells. Of those cells which are transformed, only a portion will contain the recombinant plasmid. Isolation of bacteria containing the initial plasmid and the recombinant plasmid can be accomplished if the initial vector contains a selectable genetic marker, such

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as drug resistance (e.g., antibiotic resistance). When grown on media on which only those cells containing the selectable marker can survive (e.g., on ampicillin-containing media in the case of an ampicillin resistance marker), bacteria containing the initial plasmid and the recombinant plasmid will be the only cells to survive. A further step will be necessary to isolate cells containing the recombinant plasmid (unless the DNA of interest confers a distinguishable phenotype on cells in which it occurs). This can be done, for example, by isolating and separating DNA from transformed cells and analyzing the DNA to identify cells containing the recombinant plasmids. This can be done, for example, by electrophoresis or sequence analysis.

15 After the recombinant plasmid is cloned, isolated and identified, bacterial cells in which it is included can be grown, resulting in increased numbers of the recombinant plasmid.

20 The recombinant DNA molecule can be introduced into yeast cells, using conventional procedures. Ito, H. et al., Journal of Bacteriology, 153:163-168 (1983). That cells contain the recombinant DNA molecule can be verified, both by genetic and hybridization techniques.

25 Host cells (e.g., S. cerevisiae) containing the recombinant DNA molecule are then cultured (in standard media and under standard conditions); once grown, yeast cells are stimulated by the appropriate mating pheromone. This results in induction of transcription of the yeast gene fragment and expression of the protein or 30 polypeptide of interest in substantial quantities. The protein or polypeptide is produced as part of a fusion protein, which includes the FUS1 (or FUS2) -encoded

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protein and the protein or polypeptide encoded by the DNA of interest. The protein or polypeptide is subsequently removed or released from the fusion protein; this can be done, for example, by including a cassette which encodes 05 the invertase signal sequence. This results in secretion of the fusion protein, followed by removal of the signal sequence.

Vectors containing the pheromone-inducible yeast promoter and DNA of interest can be constructed. For 10 example, a recombinant plasmid can be constructed in vitro, using known techniques. One example of such a plasmid includes: the FUS1 promoter, the initiation region of FUS1, and the gene of interest; optionally it can also contain the FUS1 signal peptide. Alternatively, 15 a plasmid can include a 5' segment or an N terminal moiety of the FUS1 gene which also encompasses DNA encoding approximately the first 254 amino acids; a gene of interest; a selectable marker (e.g., *ura3*) in yeast; a 2 micron autonomously replicating sequence (ARS); a 20 selectable marker (e.g., *amp*^R) in bacteria and a replication origin for producing the plasmid in E. coli. An example of an appropriate plasmid into which the FUS1 DNA fragment can be inserted is YEpl24.

In one embodiment of the method of the present 25 invention for expressing proteins or polypeptides of interest in yeast, a DNA fragment from either the FUS1 gene or the FUS2 gene, which includes the FUS1 or the FUS2 promoter, respectively, is fused or linked to DNA or a gene encoding the polypeptide or protein (DNA of 30 interest). The yeast DNA fragment can include nucleotide sequences in addition to those which make up the promoter sequence and, in one embodiment, also includes sequences

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encoding approximately the first 254 amino acids of FUS1. The resulting promoter-DNA of interest fusion is incorporated into a plasmid, using conventional methods. The DNA which includes the yeast promoter and additional 05 sequences and the DNA encoding the protein of interest can be produced through genetic engineering techniques (e.g., by cloning), can be synthesized mechanically or can be DNA isolated from yeast cells.

10 The FUS1 or FUS2 promoter will generally be fused or linked to the DNA or gene (i.e., with no intervening nucleotide sequences other than those which might be needed for joining the two components). However, the promoter and the DNA or gene can be separated by 15 intervening sequences of any length, provided the DNA or gene is under the control of the promoter.

18 The FUS1-gene of interest fusion or the FUS2-gene of interest fusion is inserted on an appropriate (i.e., high copy) vector, which is introduced into yeast cells by techniques known to those in the art. The vector can 20 also include a DNA sequence encoding a characteristic, such as ability or inability to metabolize a particular nutrient or drug resistance, which makes it possible to select cells into which the vector (and, thus, the promoter-DNA of interest fusion) has been introduced and 25 in which the DNA it contains is being expressed. Cells containing the vector are selected (e.g., by culturing on media containing a substance or lacking a nutrient whose presence or absence determines cells' ability to survive) and, after the cells have grown, exposed to the 30 appropriate mating pheromone.

Yeast cells containing the vector are exposed to/stimulated by the appropriate mating pheromone by the

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addition of essentially pure (either synthetic or naturally occurring) mating pheromone to the culture medium in which the transformed cells are grown, by the addition of a crude preparation of the appropriate mating pheromone, or by culturing with yeast cells of the opposite mating type (i.e., culturing a cells containing the vector with alpha cells producing alpha factor).

In one embodiment of a recombinant plasmid which can be used in the method of the present invention, the following components are included: a) a fragment of a plasmid, such as pBR322, which includes the plasmid DNA replication origin, which makes it possible to propagate DNA in E. coli; b) DNA encoding a selectable genetic marker (e.g., a drug resistance gene, such as amp^r , which makes it possible to select bacteria (E. coli) containing the recombinant plasmid; c) a fragment of the yeast 2 micron autonomously replicating sequence (ARS); d) a DNA fragment encoding a selectable genetic marker (e.g., a drug resistance gene, such as amp^r , $\text{ura}3$), which makes it possible to select yeast containing the recombinant plasmid; e) a fragment of yeast genomic DNA which includes the FUS1 promoter and sequences encoding approximately the first 254 amino acids of FUS1 and makes induction as a result of stimulation by the appropriate mating factor possible; and f) DNA encoding a protein or polypeptide not normally produced in substantial quantities in yeast cells. The latter component is positioned in the construct so as to be under the optimal control of the FUS1 promoter; induction of the FUS1 promoter leads to induction of transcription of the DNA of interest.

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The promoters and the method of the present invention, can be used to express a protein of interest, such as tpa, kpa, calf renin and bovine growth hormone. The genes encoding each of these proteins can be the 05 naturally-occurring gene or DNA encoding the protein can be synthesized. For example, as described above, the FUS1 promoter and the first 254 amino acids of FUS1 are fused, using known techniques, to the gene of interest. Maniatis, T. et al., Molecular Cloning: A Laboratory 10 Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). They are inserted onto a 2 micron high copy plasmid and introduced into a cells, again using known methods. a cells containing the plasmid and the FUS1 promoter-gene of interest fusion are stimulated 15 by addition of growing alpha cells. In this way, the a cells are stimulated by the alpha factor and induction of the FUS1 promoter occurs and leads to induction of the DNA encoding the protein of interest.

In a method for obtaining expression of a protein or 20 polypeptide of interest in yeast according to the present invention, a portion of either the FUS1 or the FUS2 gene which includes the respective promoter is fused to DNA encoding the protein or polypeptide. This is done in such a way that the DNA of interest is placed under the 25 control of the FUS1 or FUS2 promoter; preferably, it is placed under the optimal control of the promoter. The fusion produced is introduced into cells, in which the genetic information is retained and passed on to subsequent generations. Construction of a yeast strain 30 having these characteristics (a pheromone-inducible promoter linked to DNA encoding a protein or polypeptide of interest) is useful because such cells can be used

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(e.g., commercially) to produce the encoded products. Large-scale yeast fermentation methods are well developed and S. cerevisiae has no disadvantageous characteristics (e.g., toxicity).

05 In a further embodiment of the present invention, DNA encoding protein or polypeptide of interest is introduced into yeast cells through the use of retroviral vectors containing the pheromone-inducible promoter, DNA of interest and other components necessary for expression 10 of the encoded protein.

The host cell, in which the pheromone-inducible promoter-DNA of interest fusion is expressed, will generally be the yeast strain S. cerevisiae. S. cerevisiae has distinct advantages, as explained 15 previously. However, other yeast cells which can be transformed with the promoter-DNA of interest fusion can also be used.

Cultivation of yeast can be carried out under 20 well-standardized conditions; this is the case with yeast cells into which the promoter-DNA of interest fusion is introduced. For example, yeast cells can be grown on commonly used laboratory media, such as yeast nitrogen base (YNB, Difco). Stimulation of yeast cells to induce transcription of the promoter and DNA of interest can be 25 carried out in this media, with the addition of mating factor, or of yeast cells of the opposite mating type (which will produce the pheromone necessary to stimulate induction). In one embodiment of the present invention, approximately 8 micrograms (5 micromolar) of mating 30 factor is included per milliliter of culture media. If yeast cells containing the FUS1 DNA fragment - DNA of interest fusion are cultured with yeast cells of the

opposite mating type, an equal volume of each of the two types of yeasts is used. That is, cells of equal density and at the same stage of growth are cultured together; in this case, both types of cells are growing exponentially.

05 It is evident from the above description that the pheromone-inducible yeast promoter can be introduced into yeast cells, along with DNA which encodes one or more proteins of interest and transcription of which is under control of the promoter, and used to produce proteins
10 having a wide variety of applications (e.g., as drugs, enzymes, constituents of foods and beverages).

The present invention will now be illustrated with the following examples, which are not to be seen as limiting in any way.

15 Example 1 Induction of FUS1 and FUS2 by mating pheromones.

Induction of the FUS1 and the FUS2 genes was assessed as follows: For induction by alpha-factor, cells were grown to Klett 40 in YPD or SC-ura (for
20 selection of plasmids) which had been titrated to pH 4 with hydrochloric acid. Alpha-factor (Sigma Chemical Company) was added to a concentration of 5 micromolar (μ M) (1:100 dilution of a 0.5 mM solution in methanol), or methanol was added to a concentration of 1%. Cells
25 were grown for two additional hours at 30°C, at which time more than 90% of the alpha-factor treated population had arrested as shmoos or unbudded cells.

For induction by α -factor, cells were pregrown in untitrated SC-ura to Klett 40-50, and then pelleted and
30 resuspended in an equal volume of YPD media in which JY132 cells (MATA) or EEX8 cells (α /alpha) were growing

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at a similar density, or in an equal volume of unconditioned YPD. The cells were then grown for two more hours at 30°C.

05 Northern analysis. Total RNA was isolated from cells treated for 2 hours with alpha-factor or from control cells using the method of Carlson and Botstein. Carlson, M. and D. Botstein, Cell, 28:145-154 (1982). The isolated total RNA was separated by electrophoresis through a 1.5% agarose denaturing gel, transferred to the 10 nylon membrane GeneScreen Plus according to the manufacturer's instructions (New England Nuclear Research Products, Boston), and hybridized, either with labelled RNA obtained from in vitro SP6 transcription (Promega Biotech, Madison, WI) or with DNA labelled by nick 15 translation. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982). To obtain a single-stranded probe specific for FUS1, a fragment internal to the gene was inserted into an SP6 vector in the proper orientation 20 and transcribed in vitro in the presence of labelled nucleotide. The RNA probe extended from nucleotide 3635 to 4030 (see Figure 4) of the FUS1 gene. The DNA probe consisted of the BIK1-FUS1 HindIII fragment inserted into the URA3 vector YIp5. Figure 2 shows the resultant 25 autoradiogram, as well as a duplicate filter probed with the 6 kb HindIII fragment containing the entire FUS1-BIK1 region inserted into the URA3 vector YIp5. The addition of the alpha-factor pheromone to a/a cells, but not to isogenic a/alpha cells, caused an induction of a 1.6 kb 30 message not observed in cells that were not exposed to the pheromone. The Northern blot on the left in Figure 2

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is a control showing that an approximately equivalent amount of RNA was loaded onto each lane. The FUS1 gene is a prototype for the FUS2 gene. The same procedure was followed using the FUS2 gene and similar results were
05 obtained.

Beta-galactosidase assays. Cells were permeabilized by vigorous agitation in Z-buffer supplemented with 0.0075% SDS and 60 μ l/ml chloroform, and the assays were performed essentially as described by Miller. Miller,
10 J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972). Units of beta-galactosidase activity are expressed by the formula $(1000 \times O.D._{420} \text{ of centrifuged reaction mixture}) / (O.D._{600} \text{ of culture} \times \text{volume of culture} \times \text{minutes of assay})$.
15

Example 2 Construction and expression of
FUS1-heterologous DNA.

The pheromone effect described in Example 1 was analyzed further by the construction of a FUS1-LACZ fusion that had the promoter region of FUS1 and sequences which encode the first 254 amino acids of FUS1 fused to beta-galactosidase. Addition of alpha-factor to wild type a cells containing this fusion on a high copy micron vector led to at least a 1000-fold induction of
25 beta-galactosidase, as shown in Table 1.

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Table 1. Induction of FUS1-LACZ by mating pheromone.

	Uninduced	Induced*
JY132(pSB234) (<u>MAT_a</u>)	0.5	740
JY133(pSB234) (<u>MAT_{alpha}</u>)	4.7	650

05 pSB234 is a 2 micron-URA3-based plasmid which encodes the FUS1-LACZ gene product; the fusion contained the first 254 amino acids of FUS1 fused to beta-galactosidase. Units of activity were determined as described in Example 1. An equivalent induction ratio was also seen for 10 single copy derivatives of this fusion.

15 Incubation of alpha cells containing the same FUS1-LACZ plasmid with wild type a cells (a source of a-factor) also caused substantial induction; the uninduced level in alpha cells appeared to be significantly higher than in a cells. The same procedure 20 was followed using the FUS2 gene and similar results were obtained.

25 Localization of FUS1-LACZ. The cellular location of the FUS1 gene product was determined by immunofluorescence microscopy on pheromone-treated cells containing the LacZ fusion. Kilmartin, J.V. and A.E.M. Adams, Journal of Cell Biology, 98:922-933 (1984). A mouse monoclonal alpha-beta-galactosidase antibody (provided by T. Mason) was used as a probe. It was visualized using a FITC-conjugated goat anti-mouse antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN). Figure 3 shows a series of micrographs from such cells, with a CYC1-LACZ

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fusion in similarly treated cells acting as a control fusion that should be cytoplasmic. In a large fraction of the cells, the FUS1-LacZ protein appears to be located exclusively at the tip of the shmoo. The FUS2-LacZ protein was localized using similar techniques. It appears to be located within the schmoo, but not at the tip of the schmoo.

Example 3 Determination of the sequence of the FUS1-BIK1 region.

10 The 6 kb HindIII fragment containing FUS1 and BIK1 was inserted into the unique HindIII site of YCp50, in the orientation bla-BIK1-FUS1-tet. Farabaugh, P.J. and G. R. Fink, Nature, 286:352-356 (1980). The unique BamHI site of this plasmid was subsequently destroyed by 15 filling in the ends with Klenow fragment and religation of the blunt ends, generating pJef423. All complementation analyses were performed using derivatives of this plasmid.

Linker insertion mutagenesis was performed by DNase 20 I nicking, micrococcal nuclease digestion followed by digestion with S1 nuclease, and ligation of BamHI linkers, essentially as described by Shortle. Shortle, D., Gene, 22:181-189 (1983). The sequence of the BIK1-FUS1 region was obtained by inserting various 25 restriction fragments, including those obtained from the BamHI insertions, into the M13 phage derivatives mp18 and mp19, and sequencing by the dideoxy chain termination method. Biggin, M.D. et al., Proceedings of the National Academy of Sciences, U.S.A., 80:3963-3965 (1983); Sanger, F. et al., Proceedings of the National Academy of Sciences, U.S.A., 74:5463-5467 (1977).

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The precise structure of the cloned FUS1 gene was determined by dideoxy sequencing of a collection of M13 mp18 and M13 mp19 phage containing different segments of the entire region, including BIK1 sequences. The 05 sequence is presented in Figure 4. Except for small stretches of the intervening open reading frame, sequencing was carried out on both strands of the DNA. The sequence differs at four positions between nucleotides 519 and 540 from that previously determined. 10 Donahue, T.F. et al., Gene, 18:47-59 (1982). In three of these positions, an extra T was determined to occur and at one position, a T rather than an A was found. The sequence of this region is: GTAGCTGTTCATTCTCAGCGTC. The BIK1 coding region extends to within 14 bases of the 15 first upstream repeat (TGACTC) of the HIS4 gene.

The FUS1 and FUS2 genes were isolated as described below. For FUS1, this was done as follows: A yeast strain (L1052) containing a large deletion ($\Delta 453$) extending from HIS4 to LEU2 was transformed with various 20 derivatives of pJef423 harboring linker insertions. These transformants were assayed by the replica-plating test for their ability to mate successfully with a $\Delta 453$ tester lawn, L1546. Large linker-associated deletions (e.g. 550, 552; Figure 1) in the HindIII fragment 25 completely abolished the ability of the plasmid to complement the $\Delta 453$ defect; however, less extensive linker-associated deletions (e.g. 514, 543) and "simple" linker insertions (less than 50 bp deleted; 518, 483) only partially compromised the complementing activity of 30 the HindIII insert. From this mutational analysis, a portion of which is presented in Figure 1, a map was constructed which contains two distinct regions of

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complementing activity; either region alone is able to restore only partial mating competence to the $\Delta 453$ recipient.

With the exception of 565, 566, 514, and $\Delta 136$, all 05 of the mutations shown in Figure 1 were integrated into the chromosome of wild type cells (JY132 and JY133). The linker insertions were moved to URA3 integrating vectors, and the resultant plasmids were directed to integrate at chromosome III by cutting at the unique KpnI site before 10 transformation. Orr-Weaver, T. L. et al., Proceedings of the National Academy of Sciences, U.S.A., 78:6354-6358 (1981).

The recipient strains harbored the his4-34 mutation, which lies within the region of his4 that is duplicated 15 upon integration of these plasmids. Therefore, His⁺ recombinants were selected for, in order to obtain the appropriate "loop-out" excision event. Those His⁺ derivatives which had simultaneously become Ura⁻ harbored the appropriate linker insertion (or frameshifted 20 restriction site) and no plasmid sequences.

Complementation tests using these strains transformed with the original set of pJef423 derivatives confirmed the idea that two genes required for efficient mating (FUS1 and FUS2) reside on the HindIII fragment upstream 25 from HIS4.

The fus2-1 mutation was uncovered in a cross between C52a and JY145 (fus1-483). Several tetrads from this cross produced one ascospore which, when mated to $\Delta 453$ lawns, displayed a drastic defect as compared to the 30 response with wild type lawns. (fus1 alone displays only a partial defect when mated to $\Delta 453$). These segregants were outcrossed to wild type strains, and the segregation

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of wild type (good mating with all lawns), partially defective (poor mating with mutant parent lawns, but partial mating with fus1 lawns), and completely defective (poor mating with fus1 lawns and with mutant parent lawns) phenotypes among the spore clones suggested the presence of two unlinked fus mutations in the mutant parent. Single fus mutants mate poorly with double mutants, but partially with single mutants, and conversely, double mutants mate poorly with single mutants and double mutants (but mate well with wild type). The mating type of mutants does not affect their phenotype as assayed by replica plating.

The fus1 mutation was identified in strains carrying a deletion extending from HIS4 to LEU2 (Figure 1). This 15 large deletion, designated Δ 453, had no obvious effect on the vegetative growth of the cells, but subsequent genetic manipulations revealed that Δ 453 strains form diploids at greatly reduced rates with strains carrying the same deletion, but at relatively normal rates with 20 wild type strains. A centromere plasmid bearing the 6 kb HindIII fragment beginning in HIS4 and extending towards LEU2, subcloned from a 15 kb HIS4 BamHI-EcoRI fragment (isolated by "eviction"), was found to restore normal mating to a Δ 453 strain (L1052), suggesting that this 25 segment contains the mating functions missing from Δ 453. Donahue, T.F. et al., Cell, 32:89-98 (1983).

Extensive deletion analysis and random linker insertion mutagenesis (a subset of which is presented in Figure 1) reveal that the phenotype of Δ 453 results from 30 deletion of two separate genes, both of which are located on the HindIII fragment. The genes were further defined by introducing the linker insertions into the chromosome

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of wild type cells and analyzing the behavior of the resulting strains in crosses. The linker insertions fall into two groups based on complementation tests.

Insertions in one of these groups leads to a block in cellular fusion in crosses between members of the same group. The gene defined by this complementation group is called fus1. Mutants in the second group mate normally with wild type and fus1 mutants; however, in crosses with members of the same complementation group, the cells fuse normally but their nuclei fail to fuse. The gene defined by this complementation group is called bik1 (bilateral karyogamy defect). These experiments showed that the mating defect of Δ 453 results from the deletion of two genes: BIK1 and FUS1, each of which has its own unique function (see Figure 1).

Isolation of FUS2 was carried out as follows: The fus2 mutation was uncovered in crosses of fus1 by strain C52a, obtained from C. Nombela. The phenotype of fus1 mutants is rather leaky; many pairs in a fus1 x fus1 cross fuse their nuclei despite the abnormal bridge between the pair. In crosses of JY146 (alpha fus1-483 ura3-52 leu2-3, 112) by C52a (a exb1-1) several ascospore segregants were obtained which displayed a much more severe fusion defect than that of the fus1 mutant. These segregants were shown by genetic analysis to be double mutants, fus1 fus2. The fus2 mutation was apparently present in C52a, although it is not linked to the exb1-1 mutation described by Santos et al. (as assayed by the methylumbelliferyl-beta-D-glucoside overlay technique).

As shown by subsequent tests, fus2 has a phenotype similar to that of fus1.

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To clone the FUS2 gene, the severe mating defect of a fus2 mutant when mated with a fus1 fus2 double mutant was used. A FUS⁺ strain can readily mate with a fus double mutant in the replica plating assay and the fus2 defect is recessive; as a result, cloning by complementation is straightforward. A MATA fus2 strain (JY306) was used as a recipient in transformation with the Yep24 library of Carlson and Botstein. Carlson, M. and D. Botstein, Cell, 28:145-154 (1982). A clone 05 (pSB257) was identified (out of about 2000 screened) which restored normal mating function to fus2 when mated to fus1 fus2 (JY217). Genetic analysis demonstrates that the 9 kb DNA segment isolated corresponds to FUS2: 10 crosses between FUS⁺ strains and a fus2 strain harboring a FUS2⁺ -URA3⁺ plasmid integrated at fus2 yielded 19 out of 21 tetrads which contain 4 FUS⁺ spores. One-step gene 15 disruption by the substitution of an internal 1.1 kb HindIII fragment with the URA3 gene resulted in a phenotype identical to that of fus2-1: a gross mating 20 defect with fus1 fus2 strains, but only slightly reduced diploid formation with either fus2-1 or fus2::URA3 strains.

Equivalents

Those skilled in the art will recognize, or be able 25 to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A DNA fragment comprising a yeast promoter linked to DNA encoding at least one polypeptide not normally expressed at substantial levels in yeast cells, transcription of the yeast promoter inducible by a yeast mating pheromone, for directing the expression of the polypeptide-encoding DNA in yeast cells.
05
2. A DNA fragment of Claim 1 wherein the yeast promoter is a FUS1 promoter or a FUS2 promoter.
- 10 3. A DNA fragment of Claim 2 additionally comprising a nucleotide sequence encoding the first 254 amino acids of the FUS1 gene.
4. A DNA fragment of Claim 2 additionally comprising a nucleotide sequence encoding the first 617 amino acids of the FUS2 gene.
15
5. A DNA fragment of Claim 1 wherein the yeast promoter has the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4.
6. A DNA fragment of Claim 1 wherein the yeast promoter has the sequence consisting essentially of nucleotides 1 to 402 of Figure 5.
20
7. A DNA fragment of Claim 3 having the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4.

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8. A DNA fragment of Claim 4 having the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5.
9. Isolated DNA having the sequence consisting essentially of nucleotides 2550 to 3224 of Figure 4.
10. Isolated DNA having the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4.
11. Isolated DNA having the sequence consisting essentially of nucleotides 1 to 402 of Figure 5.
- 10 12. Isolated DNA having the sequence consisting essentially of nucleotides 1 to 2253 of Figure 5.
13. A yeast promoter, inducible by the appropriate mating pheromone, linked to DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 15 14. A yeast promoter of Claim 13 which is selected from the group consisting of the FUS1 promoter inducible by alpha factor and the FUS2 promoter inducible by a factor.
- 20 15. A recombinant DNA sequence which is nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.

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16. A recombinant DNA sequence which is nucleotides 1 to 2253 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 05 17. A recombinant DNA sequence which is nucleotides 2550 to 3224 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
- 10 18. A recombinant DNA sequence which is nucleotides 1 to 402 of Figure 5 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.
19. The recombinant DNA sequence of Claim 15, wherein the polypeptide is beta-galactosidase.
- 15 20. The recombinant DNA sequence of Claim 16, wherein the polypeptide is beta-galactosidase.
21. The recombinant DNA sequence of Claim 17, wherein the polypeptide is beta-galactosidase.
22. The recombinant DNA sequence of Claim 18, wherein the polypeptide is beta-galactosidase.
- 20 23. A method of expressing a protein or a polypeptide in yeast cells, comprising the steps of:
 - a. introducing into yeast cells a recombinant vector which contains a DNA fusion, the DNA fusion comprising a pheromone-inducible yeast

25

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promoter and DNA encoding a protein or a polypeptide to be expressed; and

05 b. stimulating yeast cells containing the recombinant vector with the appropriate mating pheromone.

24. A method of Claim 23 wherein the recombinant vector is a recombinant two micron yeast vector.

25. A method of Claim 24 wherein the yeast cells are wild type a yeast cells and the mating pheromone is alpha factor.

10

26. A method of Claim 24 wherein the yeast cells are wild type alpha yeast cells and the mating pheromone is a factor.

27. A protein or a polypeptide produced by the method of Claim 25, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.

15

20 28. A protein or a polypeptide produced by the method of Claim 26, wherein the DNA fusion has the sequence consisting essentially of nucleotides 2550 to 3987 of Figure 4 fused with X, wherein X is DNA encoding a protein or a polypeptide not normally expressed at substantial levels in yeast cells.

25

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29. A protein or a polypeptide produced by the method of
Claim 25, wherein the DNA fusion has the sequence
consisting essentially of nucleotides 1 to 2253 of
Figure 5 fused with X, wherein X is DNA encoding a
protein or a polypeptide not normally expressed at
05 substantial levels in yeast cells.
30. A protein or a polypeptide produced by the method of
Claim 26, wherein the DNA fusion has the sequence
consisting essentially of nucleotides 1 to 2253 of
10 Figure 5 fused with X, wherein X is DNA encoding a
protein or polypeptide not normally expressed at
substantial levels in yeast cells.
31. A protein or polypeptide produced by the method of
Claim 25, wherein the DNA fusion has the sequence
15 consisting essentially of nucleotides 2550 to 3224
of Figure 4 fused with X, wherein X is DNA encoding
a protein or a polypeptide not normally expressed at
substantial levels in yeast cells.
32. A protein or polypeptide produced by the method of
Claim 26, wherein the DNA fusion has the sequence
20 consisting essentially of nucleotides 2550 to 3224
of Figure 4 fused with X, wherein X is DNA encoding
a protein or a polypeptide not normally expressed at
substantial levels in yeast cells.
- 25 33. A protein or a polypeptide produced by the method of
Claim 25, wherein the DNA fusion has the sequence
consisting essentially of nucleotides 1 to 402 of
Figure 5 fused with X, wherein X is DNA encoding a

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protein or a polypeptide not normally expressed at substantial levels in yeast cells.

34. A protein or a polypeptide produced by the method of
Claim 26, wherein the DNA fusion has the sequence
05 consisting essentially of nucleotides 1 to 402 of
Figure 5 fused with X, wherein X is DNA encoding a
protein or a polypeptide not normally expressed at
substantial levels in yeast cells.

35. A recombinant yeast cell containing an expression
10 vector, the expression vector comprising a yeast DNA
fragment including a pheromone-inducible promoter
fused to DNA encoding a protein or polypeptide of
interest, said protein or polypeptide not normally
produced in significant amounts in yeast cells.

15 36. A recombinant plasmid comprising:
a. the FUS1 promoter;
b. DNA encoding approximately the first 254 amino
acids of the FUS1 gene;
c. DNA encoding a protein or a polypeptide of
20 interest, the DNA positioned so as to be under
the control of the FUS1 promoter;
d. DNA encoding a selectable marker in yeast;
e. a two micron autonomously replicating sequence;
f. DNA encoding a selectable marker in bacteria;
25 and
g. a replication origin for producing the plasmid
in bacteria.

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37. A recombinant plasmid comprising:
 - a. the FUS2 promoter;
 - b. DNA encoding approximately the first 614 amino acids of the FUS2 gene;
 - 05 c. DNA encoding a protein or a polypeptide of interest, the DNA positioned so as to be under the control of the FUS2 promoter;
 - d. DNA encoding a selectable marker in yeast;
 - 10 e. a two micron autonomously replicating sequence;
 - f. DNA encoding a selectable marker in bacteria; and
 - g. a replication origin for producing the plasmid in bacteria.
38. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 15.
- 15 39. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 16.
40. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 17.
- 20 41. Plasmid YEp24 having inserted therein the recombinant DNA sequence of Claim 18.

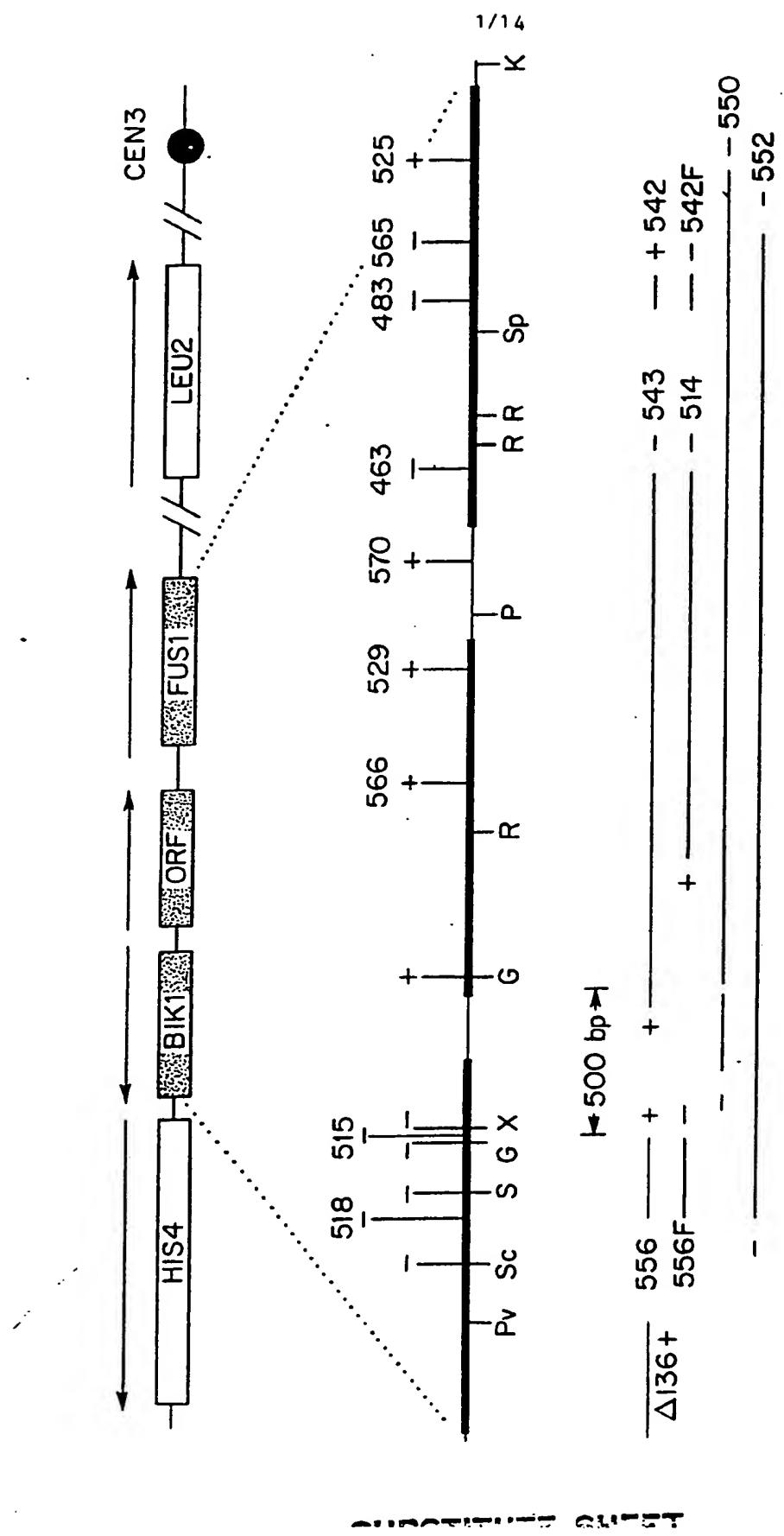
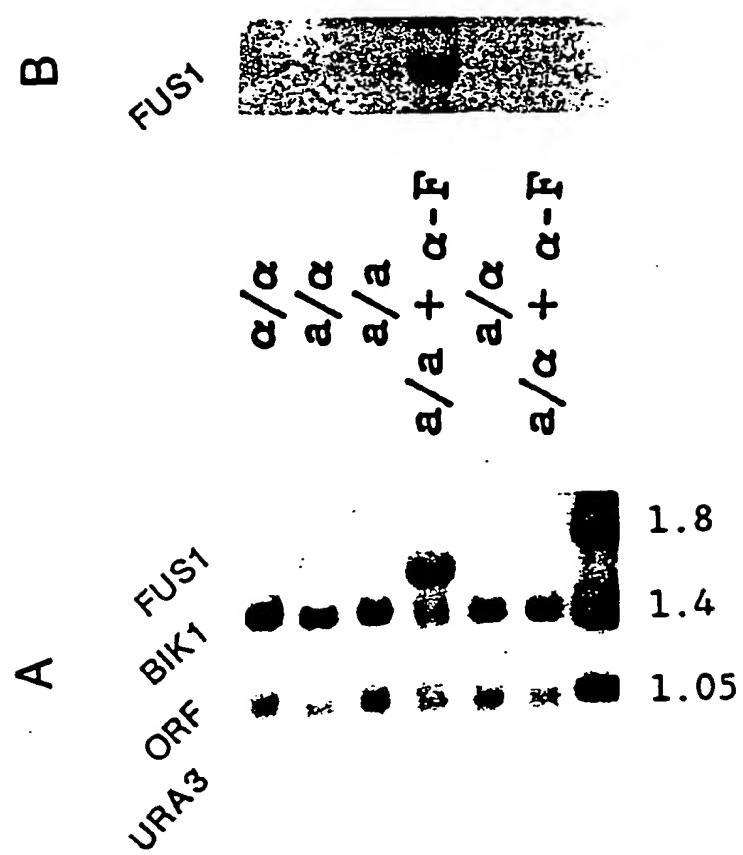


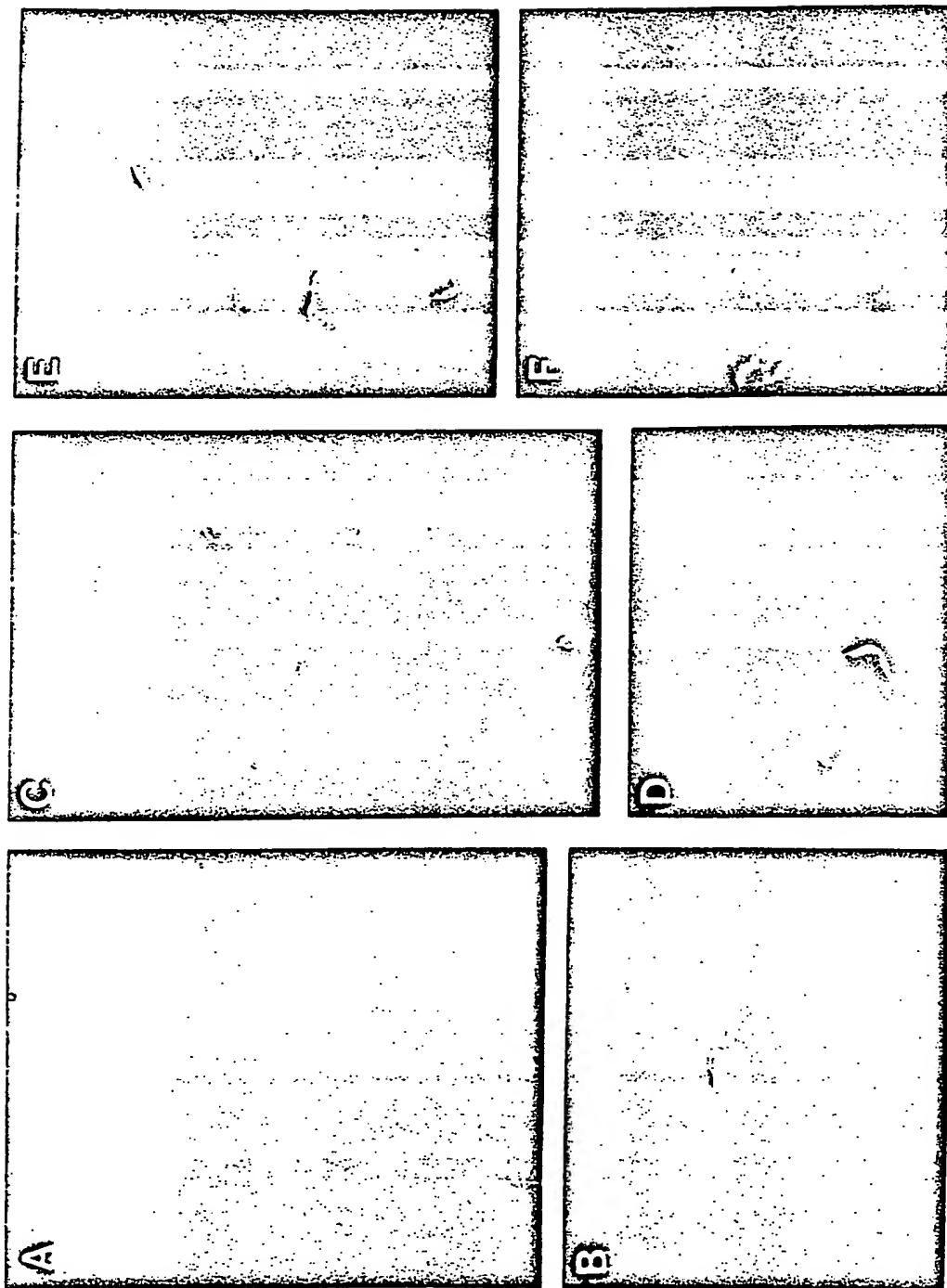
Fig. 1

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**FIG.2****SUBSTITUTE SHEET**

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FIG.3



SUBSTITUTE SHEET

FIG. 4

FIG. 4 (CONT.)

SUBSTITUTE SHEET

FIG. 4 cont'd.

FIG. 4 cont.

FIG. 4 CONT!

3000 ACTGTACCTTTGGGTGCTCATTTGGGATGAAACAAATGAAACGCTGTTATTGAAACATAACGTATTCTCGGGATTGGTTTATTAAATGACATGAAAGAG 3119

3120 TGGCTTGTAAAGGTATGTGTGCTCTTAAATATTGGATACGACATCTTTCTTAAAGCAGGATAAAAGCCATCAAGTTCTGAAATGTTGAGCAACAAATA 3239

FUS 1 → MetValAlaThrIle *

3240 ATGCCAGGACAACAACTGTGCTGACGCCAGTCGCCCAAATGTCTACTACCTTAGCATCAAATTACATACTTGCAAGCTAGTTCTCGACGGTGAACAAACAGTAACGCCAAATAGCC 3359

6 MetGlnThrThrThrValLeuThrThrValAlaLahetSerThrThrLeuAlaSerAsnTyrIleSerSerGlnAlaSerSerThrSerValThrValThrThrIleAla *

3360 ACATCAAAATACGGCTCTACCCGTCTAACTACTCTTTCTAAATGTGGGGCTCGCCAAAATCACTCTAGCAAGCACAAATGGGCTTCAATCGGACTTCCCACGGAAATATCTGTTTC 3479

46 ThrSerIleArgSerThrProSerAsnLeuLeuPheSerAsnValAlaAlaGlnProLysSerSerValSerSerIleProLysTyrIleGlyLeuSerIleGlyLeuProLysTyrIleGlyLeu *

3480 GGATTACCTATCCTTTGGTTATTCTACCTTAAAGGAATTGGGAAAGGAAATTCGAATCCAAATCCACCGCTACGATTCGCTACGATTGCGGCACTAAATGGTTC 3599

86 GlyLeuIleLeuIleCysTyrPheTyrLeuLysArgAsnSerValSerIleSerAsnProProhetSerAlaThrIleProArgGluGluTyrCysArgArgThrAsnTrpHe *

3600 TCACGGTATTTGGAGAGTAAGTGTGAGGATCAGAATTCTATCTGTTGAGAGTATTCAGACACCCAGGGACCTCGGGTCAATAACATGTCTTCAAAATAACAGTAC 3719

126 SerArgLeuPheTrpGlnSerLysCysGluAspGlnAsnSerTyrSerAsnArgAspIleGluLysTyrAsnAspTyrGlnTrpThrSerGlyAspAsnMetSerSerLysIleGlnTyr *

3720 AAAATTCCAAACCCATAATACCGCAGCATAACTGACACCTAAGAAAACGGTGAAGAACCCATACTGCTGGCTGGTAAACATTCGTTAGACCCCCAAAGTGAAACGAAATGGAGGAA 3839

166 LysIleSerLysProIleProGlnHisIleLeuThrProLysLysAsnIleSerLeuAsnProTyrAlaIlePheSerGlyLysAsnIleSerLeuAspProLysValAsnGluMetGluGlu *

FIG. 4 CONT.

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FIGURE 5 10/14

1 GATCTTTGAATTTCATTAACAGTGAAGAAGGTAGTAGAGACCGTTCAAAGTCATA 60
 10 CTAGAAAACCTAAACGTATTGTCATTCTCCATCATCTCTGGCAAAGTTCAAGTAT 30 50

 61 ACAGAGTTTAGGTAGAGGTGCCATCAGTTATTCTGACATCACTATTAATGATGGATC 120
 70 TGTCTCAAAATCCATCTCACGGTAGTCATAAGACTGTTAGTGATAAAATTACTACCTAG 90 110

 121 ATAACGATCTATTGTGCCCGCCGCGTACAAATGCGCCCCGAACCTGTCGGGAAGTTAAT 180
 130 TATTGCTAGATAACACGGGGCGCGAGTGTACCGCGGGCTTGAACAGCGCTTCATTAA 150 170

 181 CTGAAACATATATGTTACCTACTGAAACAGCGCATGTTGGAAAAGACAAAGGTGAAGACG 240
 190 GACTTTGTATATACAATGGATGACTTGTGCGTACAACCTTTCTGTTCCACTCTGC 210 230

 241 AAGTTGTATATTAAGATAGACCCTTATACATCCTTGAAAAAATTATTAATGTTGGCA 300
 250 TTCAACATATAATTCTATCTGGAAATATGTAGGAAAACCTTTTAATAATTACACCGT 270 290

 301 ACCGTCTTTATTGACAAAGTATCTTTCTTGAAACCAATTAGGTTTCTTG 360
 310 TGGCAGAAAATAACTGTTCATAGAAAAAGAACACTTGGTAAATCCAAAAGAAC 330 350

 MetPheLysThrSerTyr

 361 TTATAGTAAGTCTTAAGAAAAAGACAAGAAAACCCCTGCGATGTTAAGACTTCATAT 420
 370 AATATCATTCAAGAATTCTTTCTGTTCTTGGGAACGCTACAAATTCTGAAGTATA 390 410

 421 AsnLeuTyrAspLeuAsnTyrProLysAsnAspSerLeuThrProIleArgAspTyrLys 480
 430 AACTTGTACGATTGAACTATCCAAAAATGATTCACTAACGCCATAAGAGACTACAAA 450 470

 481 AsnAspTyrPheHisLysAsnAspAspLysLeuProGluIleValArgLysProThrArg 540
 490 AATGACTATTTCATAAAAATGATGACAATTACCAAGAAATTGTTAGAAAAACCTACGAGA 510 530
 TTACTGATAAAAGTATTTTACTACTGTTAATGGTCTTAAACAATCTTGGATGCTCT

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FIGURE 5. (CONT'D)

LysLeuSerLysHisGluAsnLysLeuAsnAspLysLysPheThrAsnLysArgProAla
 541 AAGTTATCGAAACATGAAAACAAACTCAACGATAAAAAATTCACTGAATAAACGACCAGCA 600
 TTCAATAGCTTGACTTTGTTGAGTTGCTATTTAAAGTGCTTATTGCTGGTCGT
 550 570 590

SerLeuAspLeuHisSerIleValGluSerLeuSerAsnLysLysIleTyrSerProIle
 601 AGTCTGGACTTGCAATTCTATAGTGGAGAGCCTGAGCAATAAAAAATTACTCTCCTATT 660
 TCAGACCTGAACGTAAGATACTCACCTCTCGGACTCGTTATTTAAATGAGAGGATAA
 610 630 650

AsnThrGluIlePheGlnAsnValValArgLeuAsnLeuSerProGlnIleProAsnSer
 661 AACACAGAGATTTCAAAATGTCGTGAGACTGAATTGAGCCCTCAGATTCCCATTCT 720
 TTGTGCTCTATAAAAGTTACAGCACTGACTTAAACTCGGGAGCTAAGGGTTAAGA
 670 690 710

ProHisGluGlyCysLysPheTyrLysIleValGlnGluPheTyrLeuSerGluValGlu
 721 CCTCACGAGGGATGCAAATTTATAAAATCGTACAGGAGTTTACCTCTGAAGTGGAA 780
 GGAGTGCTCCCTACGTTAAATATTAGCATGTCCTCAAATGGAGAGACTCACCTT
 730 750 770

TyrTyrAsnAsnLeuLeuThrAlaAsnAsnValTyrArgLysAlaLeuAsnSerAspPro
 781 TATTACAATAATTGTTAACCGCAAATAACGTATACAGAAAGGCATTGAATAGTGATCCA 840
 ATAATGTTATTAAACAATTGGCGTTATTGCATATGTCTTCCGTAACCTATCAGGT
 790 810 830

ArgPheLysAsnLysLeuValLysLeuAspSerSerAspGluLeuLeuLeuPheGlyAsn
 841 AGATTCAAGAATAAAACTTGTCAAGCTTGATTCAAGTGACGGAGCTATTGCTTTGGAAC 900
 TCTAAGTTCTATTGAAACAGTCGAACTAAGTTCACTGCTCGATAACGAAAACCTTG
 850 870 890

IleAspThrIleAlaSerIleSerLysIleLeuValThrAlaIleLysAspLeuArgLeu
 901 ATTGACACTATTGCGTCAATCAGCAAAACTGGTAACGCCATAAAAGACCTACGGTTA 960
 TAACTGTGATAACCGAGTTAGTCGTTATGACCATTGCCATTCTGGATGCCATT
 910 930 950

AlaLysGlnArgGlyLysMetLeuAspAlaAsnGluTrpGlnLysIleLeuThrLysAsn
 961 GCCAAGCAACGTGGAAAATGTTGGATGCGAATGAATGGCAAAAGATATTGACCAAAAT 1020
 CGGTTCTGACCCCTTTACACCTACGCTTACTTACCGTTCTATAACTGGTTTTA
 970 990 1010

FIGURE 5 (CONT'D)

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 GluValGlnGlnGlnLeuTyrSerThrPheAspIleSerGluAlaPheGluGlnHisLeu
 1021 GAGGTACAACAGCAGCTATTCAGCTATTTGATATTCAGAGGCAGTCAGCAACATTTG
 1030 CTCCATGTTGTCGTCGATATAAGTTGAAAATCTCGCAAGCTCGTTGTAAC 1080
 1050 1070
 1081 LeuArgIleLysSerThrTyrThrSerTyrPheValSerHisGlnLysGlnMetGluLeu
 TTAAGAACAAATCCACCTACACAAGCTATTTGTTAGCCACCAAAACAAATGGAACTA
 1090 AATTCTTAGTTAGGTGGATGTGTTGATAAAACAATCGGTGGTTTACCTTGAT 1140
 1110 1130
 1141 PheThrThrLeuArgMetAsnLysAsnHisPhePheAsnLysTrpTyrGluTyrCysLeu
 TTTACTACATTAAGGATGAATAAGAACATTTTTAACAAAGTGGTATGAATATTGTTA
 1150 AAATGATGTAATTCTACTTATTCTTAGTAAAAAAATGTTACCACTTATAACAAAT 1200
 1170 1190
 1201 LysGluSerGlyCysIleLysLeuGluAspIleLeuLysSerProMetLysArgLeuThr
 AAAGAGAGTGGATGTATAAGTTAGAGGACATATTGAAAAGCCGATGAAAAGACTGACT
 1210 TTTCTCTCACCTACATATTCAATCTCTGTATAACTTTGGGCTACTTTCTGACTGA 1260
 1230 1250
 1261 GlnTrpIleAspThrLeuGluSerCysTyrGluAspIleLeuSerProGlu
 CAGTGGATTGATACTTGAAACTTGGAAAGCTGTTACGAAGATATTCTTCGCCAGAA
 1270 GTCACCTAACATGAAACCTTGAACCTTCGACAATGCTCTATAAGAAAGCGGTCTT 1320
 1290 1310
 1321 LeuGlyLeuLysLeuSerProThrArgArgLysTyrSerLeuPheSerAsnLysLeuGlu
 TTGGGCTTGAAACTAACGCCCACAAGAAGAAAATATTCTTATTTCAATAAGTTAGAA
 1330 AACCCGAACCTTGATTGGCTGTTCTCTTATAAGAAATAAAAGGTTATTCAATCTT 1380
 1350 1370
 1381 ThrGluValSerGluTyrLysSerAsnSerMetTyrAsnPheSerLeuThrProSerGlu
 ACCGAGGTCTCCGAGTATAAGAGTAATTCCATGTATAATTCAAGTTAACCCCATCAGAG
 1390 TGGCTCCAGAGGCTCATATTCTCATTAAGGTACATATTAAAGTCAAATTGGGGTAGTCTC 1440
 1410 1430
 1441 IleIleGlnSerTyrAspGluAspGlnPheThrHisLeuLeuLysProProAspLysGln
 ATTATACAAAGTTATGATGAAGATCAGTTACACACCTTAAACACCCCCAGACAAACAA
 1450 TAATATGTTCAATACTACTTCTAGTCAAATGTGTGGAAAATTTGGGGTAGTCTGTT 1500
 1470 1490
 AsnLysAsnIleCysAsnAlaSerArgGlnGluSerAsnLeuAspAsnSerArgValPro

FIGURE 5 (CONT'D)

1501	AATAAAATATATGTAATGCATCTGACAAGAGAGTAATTGGATAATAGTAGAGTCCT TTATTTTATATACATTACGTAGAGCTGTTCTCTCATTAAACCTATTATCATCTCAAGGA 1510 1530 1550	13/14 1560
1561	SerLeuLeuSerGlySerSerTyrTyrSerAspValSerGlyLeuGluIleValThr TCTCTTCTTCTGGATCATCGAGTTACTACTCAGATGTATCAGGGCTAGAAATTGTCACT AGAGAAGAAAAGACCTAGTAGCTCAATGATGAGTCTACATAGTCCCCTTAACAGTGA 1570 1590 1610	1620
1621	AsnThrSerThrAlaSerAlaGluMetIleAsnLeuLysMetAspGluGluThrGluPhe AATACTTCAACTGCCCTCAGCTGAGATGATAAATCTAAAAATGGATGAAGAACAGAATT TTATGAAGTTGACGGAGTCGACTCTACTATTTAGATTTACCTACTTCTTGTCTTAAA 1630 1650 1670	1680
1681	PheThrLeuAlaAspHisIleSerLysPheLysLysValMetLysGlyLeuLeuGluLeu TTTACATTGGCAGATCACATCAGTAAATTCAAGAAAGTAATGAAAGGTTGTTAGAATTA AAATGTAACCGTCTAGTAGTCATTAAAGTTCTTCATTACTTCCAAACAAATCTTAAT 1690 1710 1730	1740
1741	LysLysAsnLeuLeuLysAsnAspLeuSerGlyIleIleAspIleSerLeuArgArgIle AAAAAGAATTATTGAAAAACGATCTGTCAGGCATTATTGATATCAGTTAACAGAAATA TTTTCTTAATAACTTTGCTAGACAGTCCGTAATAACTATAGTCAAATTCTTCTTAT 1750 1770 1790	1800
1801	AsnAlaTrpLysLysValIleGluCysGluArgProSerGlyAlaPhePheAlaHisAsp AATGCATGGAAAAGGTGATCGAGTGCACGCCCTCTGGTGCATTTTGCACCGAT TTACGTACCTTTCCACTAGCTCACGCTGCGGGAAAGACCACGTAAAAACGCGTGCTA 1810 1830 1850	1860
1861	AsnLeuIleSerThrMetCysSerSerTyrIleAspLysLeuHisGluGlnLysAsnGln AACTTAATATCGACCATGTGTTCTCGTACATAGATAAACTGCATGAACAAAAAAATCAA TTGAATTATAGCTGGTACACAAGAAGCATGTATCTATTGACGTACTGTTTTTAGTT 1870 1890 1910	1920
1921	ValThrIleLeuLysLeuThrGluLeuGluThrAspValMetAsnProLeuGluArgIle GTAACAAATTGAAACTCACAGAGCTCGAACACAGATGTGATGAACCCACTTGAAAGAAC CATTGTTAAAACCTTGAGTGTCTCGAGCTTGTCTACACTACTGGGTGAACTTCTTAG 1930 1950 1970	1980
1981	IleAlaHisCysThrThrValLysSerLysLeuLysAspLeuGlnAlaTyrMetLeuPhe ATAGCCCATTGTACTACCGTTAAAGCAAACTAAAGATTGCAAGCTTACATGTTATT 2040	

FIGURE 5 (CONT'D)

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TATCGGGTAACATGATGGCAATTTCTGGTTGATTTCTAAACGTTGAAATGTACAATAAA
 1990 2010 2030

LeuGlnGluLysLysAlaAsnValArgAspIleLysArgAspLeuLeuGlyMetHisPhe
 2041 TTACAAGAAAAAGCAAATGTGCGAGATATTAAACGTACTGTTGGGAATGCCATTTC
 AATGTTCTTTTCTGGTACACGCTCTATAATTGCACTGAACAACCTTACGTAAAG 2100
 2050 2070 2090

GlnAsnLeuGlnAsnGlnMetLysArgGluLeuProValPheIleThrLeuIleProArg
 2101 CAAACCTGCAAAACCAAGATGAAAAGGGATTACCGTCTTATTACTTGTACCCACGA 2160
 GTTTGGACGTTGGTCACTTTCCCTTAATGCCAGAAATAATGAAACTAGGGTGCT
 2110 2130 2150

TyrTyrArgMetTyrLeuValGluLeuTyrGlnSerLeuLeuLysIlePheGlyAsnHis
 2161 TACTATCGAATGTATCTGTTGAACTATATCAAAGTCTTCTAAATATTGGAAATCAT 2220
 ATGATAGCTTACATAGAACAACTTGATATAGTTCAAGAAATTATATAAACCTTAGTA
 2170 2190 2210

CysTrpTrpLysLysIleProAlaLysArgSer
 2221 TGCTGGTGGAAAAAAATACCTGCAAAAGATCTTGGAAAATATGTCTCTTAATGACTCTAT 2280
 ACGACCACCTTTTATGGACGTTCTAGAACTTTATACAGAGAATTACTGAGATA
 2230 2250 2270

AGCTACCGGCCAAATTAAAAATCTGATATTGCACTGTTATTCTAAATCACGATATAT
 2281 TCGATGGCCGGTTAATTAGAACTATAAACGTCACAATAAGATTAGTGTCTATATA 2340
 2290 2310 2330

ATGACAAAACGCATGGTAAGAAAAGATTGGCCTTCCCTGGAGACCCTAGTGGAGCCGT
 2341 TACTGTTTGGTACCATCTTCTAACCGGAAAGGGACCTCTGGGATCACCTCGGCA 2400
 2350 2370 2390

GTTGTCAGAAAACTTTCAACTTAAACAAAAGAGTATATTAGCTTATAGTTTAGAA
 2401 CAACAGTCTTTGAAAAGCTTGAAATTGTTCTCATATAATCGAATATCAAAATCTT 2460
 2410 2430 2450

TGTTTGTGTTTACTAAAGTAGTACT 2192
 2461 ACGAACACAAAATGATTCATCATGA
 2470 2490

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02129

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00, C 12 N 1/16, C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
IPC ⁴	C 12 N; C 12 P

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Molecular and Cellular Biology, volume 7, No. 2, February 1987, p 749-759, (SCOTT W. VAN ARSDELL et al), "The Yeast Repeated Element Sigma Contains a Hormone-Inducible Promoter".	1-4, 13-14, 23-26, 35, 36
P, Y	Chemical Abstracts, Vol 107 (1987) abstract No 212743x, (Trueheart), Mol.Cell.Biol.1987, 7(7), 2316-28 (Eng). *Abstract*	1-41
P, Y	Chemical Abstracts, Vol 107 (1987) abstract No 128287u, (McCaffrey) Mol.Cell.Biol. 1987, 7(8), 2680-90 (Eng). *Abstract*	1-41
P, Y	Chemical Abstracts, Vol 108 (1988) abstract No 217221f, (Nonato, Roberto V.) Biochem.Biophys.Res.Commun. 1988, 152(1), 76-82 (Eng). *Abstract*	1-4, 13-14, 23-26, 35, 36
Y	EP, A2, 0 183 350 (IMMUNEX CORPORATION) 4 June 1986 *Claim 7*	1-4, 13-14, 23-26, 35, 36
		.../...

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

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"D" document referring to an oral disclosure, use, exhibition or other means

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

23th September 1988

Date of Mailing of this International Search Report

11 NOV 1988

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

P. G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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A	Chemical Abstracts, Vol 104 (1986) abstract No 220092n, (Vlasuk George P) J. Biol.Chem. 1986, 261(11), 4789-96 (Eng). *Abstract*	1-41
A	Chemical Abstracts, Vol 104 (1986) abstract No 16037u, (Singh Arjun et al) Genet:New Front., Proc.Int.Congr., 15th 1983 (Pub.1984). 2, 33-9 (Eng). *Abstract*	1-41
A	US, A, 4 615 974 (KINGSMAN et al) 7 October 1986 *The whole document*	1-41

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/02129

SA 23281

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EPO file on 01/09/88
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EP-A- 0123544	31-10-84	JP-A- 60041487	05-03-85
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